Exploring molecular signatures of N, N-Dimethyltryptamine pathway in neural cells and their convergence into cognition

A

Synopsis

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INTRODUCTION

Cognition is the mental process involved in attention, perception, memory, language, learning and higher reasoning. It is a function of biological, physical, chemical components of cellular machinery. These processes work in cohesion to generate higher attention span and related cognition processes. Chemical component ensures well-orchestrated reactions of neuro -chemicals that also provide energy transfer between molecules. The energy transfer includes electron movement among neurochemicals. Amongst the biological components involved neural cells have been demonstrated to carry out all three reaction systems, biological, chemical and physical in a coordinated manner.Till recently neuroscience advocated primary importance to neuronsfor signal generation and transmission of cognitive signals to different parts of brain.In the last decade neuron associated glial cells and astrocytes have been found to play critical role in neuro cognitive processes.Various functional aspects of glial cells and astrocytes have been identified that permit them to relay neural information at speed, time, and directionof information functionalization. The concept of tripartite system highlights direct role of the prolific neuron-astrocyte signaling in synaptic function (Perea et al., 2014).

Among the significant biochemical pathways involved in neurocognitive regulation, Tryptophan pathway with its neuroactive metabolites like serotonin, melatonin and tryptamine trigger supernatural perception and influence cognitive mental states.Serotonin (5-HT) is a crucial monoamine neurotransmitter in central nervous system (CNS) and central to melatonin biosynthesis in the pineal gland (Höglund et al., 2019). Several neurodegenerative conditions, particularly Alzheimer's Disease and Parkinson's Disease, are characterized by more irregular circadian rhythms and lower melatonin content than in age-matched controls (Zhang et al., 2017). Tryptamine and DMT are endogenous trace amines with low concentration in CNS but play significant role as neuromodulators. N, N-Dimethyltryptamine (DMT) is the simplest tryptamine, endogenous serotonergic psychedelic and can cross blood brain barrier (Barker, 2018).It procures a spectrum of subjective effects on brain functions including perception, cognition and exhibits antihypoxic, antidepressant and plasticity promoting actions. Its rapid metabolism and availability in trace amounts makes detection, mapping, characterization and scientific studies strenuous (Szabo et al., 2016).

DMT is referred to as ‘Spirit Molecule’ because of its deep psycho-spiritual entheogenic properties. It is believed that DMT uniquely reprograms the brain circuitry allowing self-consciousness to separate from body consciousness while remaining aware (Strassman, 2001). Neural correlates of the DMT experiences described as world analogous assessed with multivariate EEG showed reduced oscillatory power in alpha and beta bands and robustly increased spontaneous signal diversity (Timmermann et al., 2019). The phenomenology of DMT experience suggests it may be a powerful potential neuro-pharmacological target and experimental tool for illuminating the neurobiology of consciousness, psychedelic neurotransmitter pathways and cognitive states. Regulatory pathways, polyfunctional neuronal circuits and associated cellular activity are governed by the fluctuations in gene expression and protein synthesis at molecular level. These molecular signatures are the neural representations that map the different mental states in brain(Hansen et al., 2020).

Pineal gland in a photo-neuro-endocrine organ that secretes serotonin, melatonin and DMT (Gheban et al., 2019). Pineal gland is popularly called as ‘Tisra Til’ or ‘Third Eye’ by eastern religions. Strassman hypothesized pineal gland to be the DMT factory because it is rich in all the enzymes and biochemical machinery necessary for DMT production i.e. has rich network of sympathetic, parasympathetic and some non-myelinated fibersfrom CNS and trigeminal ganglia,a rich blood supply, presence of methyltransferase enzymes and beta-carboline,unique position at junction of third ventricle, around center of the brain bathing in cerebrospinal fluid and therefore capable of triggering rapid communication through DMT (Strassman, 2001;Cameron & Olson, 2018).Recent studies have shown colocalization of key DMT synthesis enzymes i.e. INMT-positive and AADC-positive cells in the pineal gland, cerebral cortex, hippocampus and choroid plexus (Dean et al., 2019).SCN and Pituitary Pars Tuberalis have demonstrated melatonin driven entrainment of circadian rhythms and regulation of seasonal physiology(Johnston & Skene, 2015). DMT alters cortical travelling waves as observed with striking changes in cortical dynamics in eyes-closed DMT, which are remarkably similar to those observed during actual eyes-open visual stimulation (Alamia et al., 2020).

To dissect cellular compositions and characterize molecular properties of these complex organs and tissues, high throughout put single cell RNA sequencing, bioinformatics coupled with genomics, transcriptomics and proteomics has emerged as a revolutionary approach(Haque et al., 2017).Single-

cell RNA-sequencing (scRNA-seq) is an exciting and cutting-edge method for analyzing differences in cellular gene expression particularly for tissue heterogeneity analyses, lineage tracing, and cell population dynamics. Insilico scRNA seq data analysis spans a range of functions from quality control assessment, filtering of unwanted genes and cells based on various quality metrics, trimming, alignment, quantification, batch correction, normalization, dimensionality reduction and clustering into distinct cell subtypes or for inferring pseudo-temporal trajectories and assessing differential gene expression specific for each cell subtype (Ji & Sadreyev, 2019). Pathway analysis and gene regulatory networks can also be studied in depth by integration of miRNA analysis from the differentially expressed genes, thus building a miRNA regulatory network (L. Chen et al., 2019).

From this proposal we wish to delve towards providing concrete scientific proofs by exploring molecular signatures of DMT pathway in neural cells with convergence to cognition. Comparative analysis of Serotonin, Melatonin and DMT will provide better understanding of their role, mechanism and regulatory pathways. Therefore, establishing an interactome between the overlapping neurologically significant pineal gland, suprachiasmatic nucleus, pituitary gland and cerebral cortex involved in tryptophan metabolism could highlight the biochemical pathways and molecular dynamics involved supernatural perception and cognitive states. Linking it to various studies concerned with cognitive behaviour, consciousness and EEG experiments would widen the scope of study providing an interdisciplinary outlook. Modelling this study in human and rodents will add to the evolutionary perspective. Thus, this *insilico* analysis will highlight human brain neural coordinates with respect to DMT pathway and open new avenues for neurobiological research as well as clinical and pharmaceutical research applications.

REVIEW OF LITERATURE

This interdisciplinary study explores molecular signature of DMT pathway in neural cells and their convergence to cognition. Therefore, the review of literature is divided into three distinct sections-

(i) Tryptophan Metabolic Pathway and Dimethyltryptamine

(ii) Cognition and Neuro-glial network

(iii) Molecular signature and Omics

I. TRYPTOPHAN METABOLIC PATHWAY AND DIMETHYLTRYPTAMINE

L-Tryptophan is an essential amino acid that is important for production of several biologically active metabolites, including many important neuromodulators of central nervous system(CNS) which exert profound effects on cognition and physiological processes(Ruddick et al., 2006). An increase in the plasma Tryptophan/Large neutral amino acids ratio increases tryptophan influx to the brain. Displacement of albumin bound tryptophan by non-esterified fatty acids increases availability of free tryptophan and is dependent on cerebral hemodynamics with greater dissociation under low cerebral blood flow(Richard et al., 2009).

Once tryptophan has crossed the blood brain barrier, it is freely available for uptake by cells of CNS for utilization in multiple metabolic pathways.Tryptophan undergoes extensive metabolism along following pathways- Serotonin and Melatonin are major products from hydroxylation, Tryptamine and its methylated forms result from decarboxylation, Indolepyruvic acid from Transamination and Kynurenine and other metabolites from Oxidation pathway(Höglund et al., 2019).

a) Major neuroactive Tryptophan metabolites -

(i) **Serotonin**

Serotonin (5-HT) is a crucial monoamine neurotransmitter in central nervous system (CNS) and an important hormone in peripheral tissues. Tryptophan serves as the precursor, it is hydroxylated to 5-hydroxytryptophan (5-HTP) by Tryptophan hydroxylase (TPH),the rate limiting enzyme andsubsequently decarboxylated to Serotonin (5-HT) by (AADC) amino acid decarboxylase. Two isoform of TPH are known, TPH1 in the periphery and TPH2 in the CNS(Nichols & Nichols, 2008).

Serotonin is involved in appraisal and stress coping mechanisms, modulation of behavioral and neuroendocrine responses to stressors. During postnatal life, there is increase in numbers of serotonin containing axons in the suprachiasmatic nucleus (Takeuchi et al., 2014).A high density of serotonergic projections in hippocampus and prefrontal cortex underlies the anatomical and neurochemical linkage of serotonergic system with brain areas associated to learning and memory (Badawy, 2018).

Serotonergic class of psychedelics like DMT act as partial agonists of serotonin receptors, primarily the 1A,2A and 2C receptors ,with predominant interest in serotonin 2A receptor ($5\text{-}HT_{2A}$) (Burbach et al., 2018).The phasic effects of psychedelics include stimulation and enhancement of serotonin;

saturation and overloading of serotonin system; and release of the habitual serotonin repression of the dopaminergic system(Canal, 2018).Serotonin serves as a precursor for production of 5-hydroxy-dimethyltryptaminewhich is analog of DMT (Barker, 2018). Serotonin is also central to melatonin biosynthesis in pineal gland (Lv et al., 2020).

(ii) Melatonin

In Melatonin biosynthesis from tryptophan, serotonin is the main intermediate product. N-acetylation of serotonin to N-acetylserotonin (NAS) is catalysed by Serotonin N-acetyltransferase (SNAT), the rate-limiting step in melatonin synthesis. Further methyl group transfer from S-adenosyl-L-methionine onto N-acetyl-serotonin is catalysed by hydroxyindole-O-methyltransferase (HIOMT) to produce Melatonin (Tan et al., 2003).Melatonin has a typical circadian pattern of low levels during the daytime, while the propensity increases at night. Organization of circadian rhythm in mammals acquire a primary linear route which starts at reception of environmental photic signals in eyes (retinae), thereby via retino-hypothalalamic tract (RHT) being transmitted to the suprachiasmatic nuclei (Hickie et al., 2013). Pituitary Pars Tuberalis also drives the photoperiodic control over multiple aspects of neuroendocrine physiology (Johnston & Skene, 2015).

The decrease of melatonin receptors with senescence, pineal gland calcification or dysfunction is seen to be associated with neurological diseases and other pathologies. Alzheimer's Disease (AD) and Parkinson's Disease (PD), are characterized by more irregular circadian rhythms and lower melatonin content than in age-matched controls(D. Zhao et al., 2019).

(iii) Tryptamine and N, N- Dimethyltryptamine (DMT)

Tryptamine and DMT are endogenous trace amines with low concentration in CNS and play significant role as neuromodulators. Tryptamine is produced by decarboxylation of tryptophan catalyzed by aromatic L-amino acid decarboxylase (AADC) which uses pyridoxal 5'-phosphate (PLP) as cofactor and releases CO_2(Szabo et al., 2016; Badawy, 2019). Brain tryptamine in humans and several animal species is generally in the sub-micromolar range, but can be dramatically increased by MAO inhibition.

- **N,N-Dimethyltryptamine**

DMT has been popularly referred to as the 'Spirit molecule' to implicate the mystical experiences

which can lead to spiritual realms, induce altered state of consciousness, out of body and near death experience (Strassman, 2001). DMT is attributed to be a neurotransmitter in the mammalian brain (Frescka, 2013). DMT is the simplest tryptamine and a naturally occurring serotonergic psychedelic capable of causing wide spectrum of subjective effects on brain functions including perception, cognition and antihypoxic, antidepressant and plasticity promoting actions. DMT is among the few select molecules that can cross the blood brain barrier(Szabo et al., 2016). Like serotonin and melatonin DMT is biosynthesized from tryptophan. Decarboxylation of tryptophan by aromatic L-amino acid decarboxylase (AADC) into tryptamine and its subsequent N, N-dimethylation by indolethylamine-N-methyltransferase (INMT) with S-adenosyl-l-methionine as the methyl source leads to production of DMT. Indoleacetic acid (IAA) is primary metabolite obtained from metabolism of DMT via monoamine oxidase A (MAO-A) (Cameron & Olson, 2018)

Knowledge about synthesis, biodistribution and quantitative levels of DMT in blood, urine and even CSF of humans is very limited.LC/MS/MS analysis in pineal gland microdialysate shows presence of DMT(Barker, 2018). Endogenous DMT under normal physiological conditions is found in trace amounts. Due to its rapid metabolism in periphery into inactive metabolites quantification of threshold for causing a meaningful biological effect or psychedelic experience is difficult to assess.It is not orally active, until co-administered with a monoamine oxidase inhibitor (MAOI).In the *in vivo* metabolism studies, DMT is pharmacologically active following administration by injection (intravenous or intramuscular routes) or smoking (vaporization and inhalation), pathways which can avoid first-pass metabolism by the liver to some degree. The time of onset of effects is rapid (seconds to minutes) by these routes is short depending on dose and route(Mcilhenny et al., 2011; Rodrigues et al., 2019).

DMT uptake in neuronal cells is thought to be by serotonin uptake transporters (SERT) on neuronal plasma membrane, while its sequestration into synaptic vesicles from the cytoplasm by the neuronal vesicle monoamine transporter 2 (VMAT2).DMT has been shown to interact with a variety of ionotropic and metabotropic receptors(dos Santos et al., 2017). The activation of frontocortical glutamate receptors, secondary to serotonin 5-HT2A receptor-mediated glutamate release, appears to be a controlling mechanism of serotonergic hallucinogens. The structural plasticity occurs via mTOR

dependent mechanism that involves activation of 5-HT2A receptors(Carbonaro & Gatch, 2016). DMT acts as agonist of trace amine-associated receptors (TAARs). It also has affinity for sigma-1receptor, regulatessecretion of brain derived neurotrophic factor (BDNF) and stress response in endoplasmic reticulum. Using *in vitro* cultured human cortical neurons DMT-mediated sigma-1 activation may alleviate hypoxia-induced cellular stress via decreased expression and function of the stress factor HIF-1α in severe hypoxia (Szabo et al., 2016).

To study neural correlates of DMT by bolus intravenous injection of DMT to showed oscillatory power of frequency bands and signal diversity in human brain activity recorded by multivariate EEG and may find significance in neurobiological underpinnings of immersive states of consciousness(Timmermann et al., 2019).

b) Regulation of Tryptophan Biosynthetic pathways : A coordinated regulation of Serotonin, Melatonin and Dimethyltryptamine

Tryptophan is metabolized along the kynurenine, serotonin and tryptamine pathways resulting in formation of kynurenine metabolites, serotonin, melatonin, tryptamine and dimethyltryptamine etc.(Gomes et al., 2014). Major difference between the pathways is that only kynurenine pathway leads to loss of indole moiety while others preserve it. Serotonin pathway is assumed to shift to kynurenine pathway under stress due to increase in proinflammatory cytokines which activate IDO, the extra-hepatic rate limiting enzyme for kynurenine formation(Li et al., 2017).

Tryptophan being the precursor of all the different metabolites, what conditions trigger the secretion of one metabolite against the other and if thereare specific cell types for uptake of tryptophan in CNS is unknown. Pineal gland has the ability to produce both serotonin and DMT butwhat determines the shunting mechanism and whether the selectivity on basis of presence of the rate limiting key enzyme sufficient to describe the interplay. INMT (indolethylamine-N-methyltransferase) and AADC (amino acid decarboxylase) are key biosynthetic enzymes for DMT production. INMT mRNAis colocalized along with AADC in pinealocytes, cerebral cortex, hippocampus and choroid plexus in rat. INMT-positive and AADC-positive neurons may function as DMT producing neurons. However, in pineal gland AADC along with TPH is well known to mediate

synthesis of serotonin. AADC has also been reported in cerebral cortex and hippocampus neurons that do not contain TPH and the function of these neurons is unknown till date (Dean et al., 2019).

Serotonin is a precursor for both melatonin and 5-hydroxy-DMT. Due to similarity and overlap of functions, differentiation by cell is an important aspect that needs to be addressed. For both serotonin and DMT, 5-HT2A receptor is of primary importance. How and where the competitive binding occurs A study demonstrated the functional that the 5HT2A receptor induced head twitch response in mice via the Beta-Arrestin/Src/Akt signaling in frontal cortex and primary cortical neurons is unique to serotonin selectivity between serotonin and psychoactive N-methyltryptamine (Schmid & Bohn, 2010).

For coordinated regulation of serotonin, melatonin and DMT, the common interactory organs i.e. the pineal gland, pituitary gland, cerebral cortex and suprachiasmatic nucleus overlap in biosynthesis and metabolism and thereby might find significant functional correlation to explain the interplay and role in cognition.

II. COGNITION AND NEURO-GLIAL NETWORK

Cognition refers to all the processes and activities concerned with acquisition, storage, retrieval and processing of information. The cognitive information coding, processing and transmission to other cells is done by brain coding elements- neural cells and glia. While traditional neuroscience has advocated a larger importance to neurons, but new scientific evidence has provided impetus to equal importance of glial cells as integral players. Astrocytes are highly abundant glial cells in the brain and play active role in neurotransmission, neurophysiology, metabolic and homeostatic functions of the nervous system. Han et al. transplanted human glial progenitor cells into mouse forebrains which gave rise to smarter mouse with human astrocytes as it displayed improved long term potentiation and learning (Y. Zhang & Barres, 2013)To highlight the prolific neuron-astrocyte signaling the concept of ‘tripartite synapse’ signifying direct role of astrocytes in synaptic function by uptake of neurotransmitters and release of neuroactive substances i.e. gliotransmitters was introduced(Perea et al., 2014).

Cognitive processes arise from coherent coordinated activity of neuro-glial network exerting global effect on network ensemble, neurocognitive functions and molecular dynamics of brain

processing(Mederos et al., 2018). Multiscale integration of astrocytes with synaptic and neuronal networks may possibly occur at nano, micro, syncytium and meso spatial scales of interaction (Santello et al., 2019). Abnormalities of astrocyte-neuron signaling in prefrontal cortex due to reduced glial release of ATP are related with depression like behaviour affecting cognitive function such as attention, reversal learning functions and working memory (Lima et al., 2014). Dynamic change in neuroglial network within the hypothalamus –hippocampus circuit cause central fatigue and cognitive dysfunction in presence of neuroactive tryptophan metabolites specifically tryptophan-kynurenine pathway (Yamashita, 2020).

III. MOLECULAR SIGNATURE AND OMICS

The human brain is an integrated dynamic vibrational system having neural representation of intricate information processing through multiscale interactions across gene to molecular level (Hansen et al., 2020). How unique patterns of gene expression manifest a defined cognitive output remains a key question in cognitive neuroscience. At the molecular level, fluctuations in gene expression and protein synthesis in neurons drive single-cell activity. The activation-inhibition of cellular activity promotes synaptic remodeling shaping the wiring of nested and increasingly polyfunctional neural circuits (Mederos et al., 2018).

Genomics is the study of organisms' whole genomes (WGS).Transcriptomics is the study of transcriptome, the complete set of RNA transcripts produced by the genome at any one time (Manzoni et al., 2018). It is specifically focused on how transcript patterns are affected by development, disease, or environmental factors such as hormones and drugs. Recent advances in genome wide sequencing technology from microarray technology to RNA sequencing ,cell isolation techniques, and genetic array have made possible an unbiased interrogation of the transcriptomic studies with greater precision and granularity (Mu et al., 2019). MicroRNAs (miRNAs) also play significant role in regulation of post-transcriptional expression of protein-coding genes involved in most of the cellular processes.

Coupling bioinformatics with genomics and transcriptomics enhances analysis speed, data coverage and supports better understanding of biological system. Insilico analysis of single cell RNA sequencing (scRNA-seq) data will help identify rare cell populations, cellular gene expression, trace

lineage and developmental relationships between heterogeneous yet related cellular states (G. Chen et al., 2019).Typical bioinformatics workflows for scRNA-seq analysis include the following major steps: (1) Quality control (QC)assessment and filtering of unwanted genes and cells based on various quality metrics, trimming adapters, primers or overrepresented sequences; (2) normalization of gene expression and estimation of dispersion for expression values for all genes; and (3) identification of cells with similar gene expression patterns for clustering into distinct cell subtypes or for inferring pseudo-temporal trajectories. This would help in the identification of gene expression signatures i.e. differential gene expression specific for each cell subtype (Ji & Sadreyev, 2019).

Seurat (Satija et al., 2018) is designed for QC, analysis, and identification of distinct subpopulations of cells within scRNA-seq datasets; FastQC for quality control and Cutadapt for trimming. Transcripts can be aligned to the genome or the transcriptome (Skinnider et al., 2019) and is usually faster when dealing with well-annotated species such as *Homo sapiens* or *Mus musculus*. The application of dimensionality reduction methods such as PCA or t-distributed stochastic neighbour embedding (tSNE) to the refined gene expression counts, in order to identify and visualize subgroups has been widely applied to the visualization of scRNA-seq results (Haque et al., 2017; X. Zhao et al., 2017). Single-cell RNA sequencing of the mammalian pineal gland identified nine transcriptionally distinct cell types ~90% were classified as melatonin-producing α- and β-pinealocytes (1:19 ratio). Non-pinealocytes included three astrocyte subtypes, two microglia subtypes, vascular and leptomeningeal cells, and endothelial cells (Mays et al., 2018).

The miRBase database is an online repository with a searchable interface which stores microRNA nomenclature, sequence data, annotation and target predictions.TarBase and mirWalk stores information of miRNA targets in human, mouse, fruit fly, worm, and zebrafish (Sethupathy et al., 2006 ; L. Chen et al., 2019). The database is functionally linked to Gene Ontology (GO) and UCSC Genome Browser. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a comprehensive knowledge base for both functional interpretation and practical application of genomic information (Kanehisa et al., 2017). STRING, DAVID, Cytoscape and KEGG are used interchangeably in combinations for pathway analysis (Milano et al., 2019).BrainEXP (Jiao et al.,

2019),BrainMap and other relevant softwares for brain functional connectivity may be linked to neurocognitive behaviour by mapping to MRI scans and other experimental data.

OBJECTIVES

The aim of this study is to explore the molecular signatures of serotonin, melatonin and DMT on neural cells and astrocytes by analysis in pineal gland, pituitary gland, suprachiasmatic nucleus and cerebral cortex of human and rodent. This will highlight the human brain neural coordinates with respect to DMT pathway in convergence to cognition.

1. **Identification of neural cell types** in Human and Rodent –
    a) Pineal gland – Pinealocytes and astrocytes
    b) Pituitary gland- Pituicytes, neural cells and astrocytes
    c) Suprachiasmatic Nucleus- neural cells and astrocytes
    d) Cerebral cortex - neural cells and astrocytes
2. Identification of **differentially expressed genes (DEG)/transcript abundance** in neural cell types of Pineal gland, Pituitary gland, Suprachiasmatic Nucleus and Cerebral cortex**relative to enzymes of DMT Pathway**
3. **Extraction of miRNA** from the curated differentially expressed genes (DEG) in neural cell types of Pineal gland, Pituitary gland, Suprachiasmatic Nucleus and Cerebral cortex
4. **Pathway construction** to establish a **connectome** between Pineal gland ,Pituitary gland, Suprachiasmatic Nucleus and Cerebral cortex and their **comparative analysis in Rodent and Human**
5. **Correlation with neurocognitive behaviour** /states and consciousness processing

MATERIALS AND METHODS

MATERIALS-

Bioinformatic analysis will rely on software. List of bioinformatics software which be updated with time as per evolution of softwares and as per need of the objectives.

Table 1: List of important bioinformatics softwares-

GEO(Gene Expression Omnibus) Datasets	https://www.ncbi.nlm.nih.gov/geo/	RNA Seq processed data
SRA(Sequence Read	https://trace.ncbi.nlm.nih.gov/Traces/sra	RNA Seq raw datasets

Archive	/	
Reference genome	NCBI	
miRNA Seq database	MirGeneDB, miRBase	
		PRE-PROCESSING
	SAM & BAM tools	Format conversion
	Trimmomatix ,FASTX	Adaptor trimming
	HISTAT2,STAR,Tophat2	Read Alignment
	Cell Ranger, Kallisto, Cufflinks	Read counting
		QUALITY CONTROL(QC)
	FASTQC	QC on sequences
	RNASeQC QC	On read counts
	GATK	QC recalculation/variant calling
		CONFOUNDING EFFECT CORRECTION
	SCnorm, scran,SCATER	Normalization
	KBET, MNN	Batch EC
		DOWNSTREAM ANALYSIS
	tSNE, PCA	Dimensional reduction
	Seurat	Feature selection
	Seuret,ZIFA,SC3	Clustering
	CuffDif,EdgeR,DESeq2	Differential gene expression
	Monocle, TSCAN, CellRouter	Trajectory Inference
	Census, BRIE	Alternative splicing
	SCALE	Allelic expression
	SCENIC	Network Inference
	mirWalk	miRNA binding site
	DIANA- TarBasev8	Validated miRNA-gene interaction
	DIANA miRPath	miRNA pathway analysis
	BrainSpan	Developmental transcriptome brain
	NeuroSynth	fMRI data brain
	BrainMap	Neuroimaging database of brain
	REACTOME	Pathway database
	DAVID, KEGG	Pathway analysis
	STRING	Protein association networks
	TargetScan	MiRNA target prediction
	miRDeep	Integrated miRNA tools
	CytoScape, jActiveModule	Network analysis
	GALAXY	NGS platform

METHODS-

The methodology corresponding to each objective is as follows-

1. **Objective - Identification of neural cell types in Pineal gland, Pituitary gland, Suprachiasmatic Nucleus and Cerebral cortex-**

a) Data extraction

For identification of pinealocytes and astrocytes in Pineal gland, neural cells and astrocytes in Pituitary gland,Suprachiasmatic nucleus and Cerebral cortex, the single cell RNA-Seq reads will be extracted from the Sequence Read Archive (SRA NCBI) and the NCBI Gene Expression Omnibus (GEO)

Extraction of Reference genome/transcriptome (GRCh38)- Human genome (*Homo sapiens*) and Rodent (*Mus musculus*) from NCBI.

b) Quality control and Trimming

To avoid misinterpretation and bias in data due to sequencing errors, sequence quality control is an essential step. It will be performed using softwares like FastQC to createa report of sequence quality, by MultiQC, Cutadapt or Trimmomatix to improve the quality of sequences via trimming and filtering (He et al., 2020).

c) Mapping/Alignment

Mapping will be done by bioinformatics softwares like STAR, HISTAT, salmon or Tophat2. Spliced-awared mappers help to efficiently map transcript-derived reads against a reference genome/transcriptome. Stringtie may also be used if required (Peri et al., 2020).

d) Post Alignment Quality control and Normalization

Post alignment quality control will be done by bioinformatics softwares like RNASeQC to rectify for further downstream analysis. Normalization enables consistent comparison across cells, including technical variation and impact by outliers. SCnorm, scran or Linnorm will help in normalization (Hie et al., 2020).

e) Quantification of mapped reads

Quantification will be done by softwares like HTSeq-count or featureCounts at gene level and Kallisto and Cufflinks at transcript level (Jin et al., 2017).

f) Dimensional Reduction and Visualization

Dimensional Reduction is used to enhance prediction rate and reduce the number of random variables by obtaining a set of principal variables (Meng et al., 2016).PCA, t-SNE and similar bioinformatics will be used.

g) Clustering

Clustering is used to separate cells into groups to describe heterogeneity between samples. It can be both supervised and unsupervised. Marker genes may be identified for clustering analysis. For clustering SEURET, ZIFA or SC3 will be used (Hwang et al., 2018).

2. Objective- Identification of differentially expressed genes (DEG)/transcript abundance in neural cell types of Pineal, Pituitary, SCN and Cerebral cortex relative to enzymes of DMT Pathway

The number of sequenced reads mapped to a gene depends on sequencing depth of the samples and length of the gene. Transcript abundance for the key enzymes that regulate Serotonin, Melatonin and DMT synthesis when mapped to the human and mouse transcriptome will help uncover the transcript abundance indicating their significance in neural cell types.

DESeq2 is a great tool for dealing with RNA-seq data and running Differential Gene Expression (DGE) analysis. Differential expression signature can be visualized via interactive graphics that include heat map ranked by false discovery rate (FDR), log fold change vs. log average expression (MA) plot and gene detectability plot. Also Monocle 3, MAST or SCDE may be used (Yin et al., 2019; S. Zhao et al., 2016).Gene enrichment may also be carried out.

3. Objective- *Insilico*extraction of miRNA from the curated differentially expressed genes

MicroRNA extraction from the curated differentially expressed genes will be done by miRwalk, mirDB, TargetScan. Also if miRNA-sequencing raw reads are available from database, they can be processed and by edgeR differentially expressed miRNAs may be obtained (L. Chen et al., 2019).

4. Objective- Pathway construction to establish connectome and comparative analysis

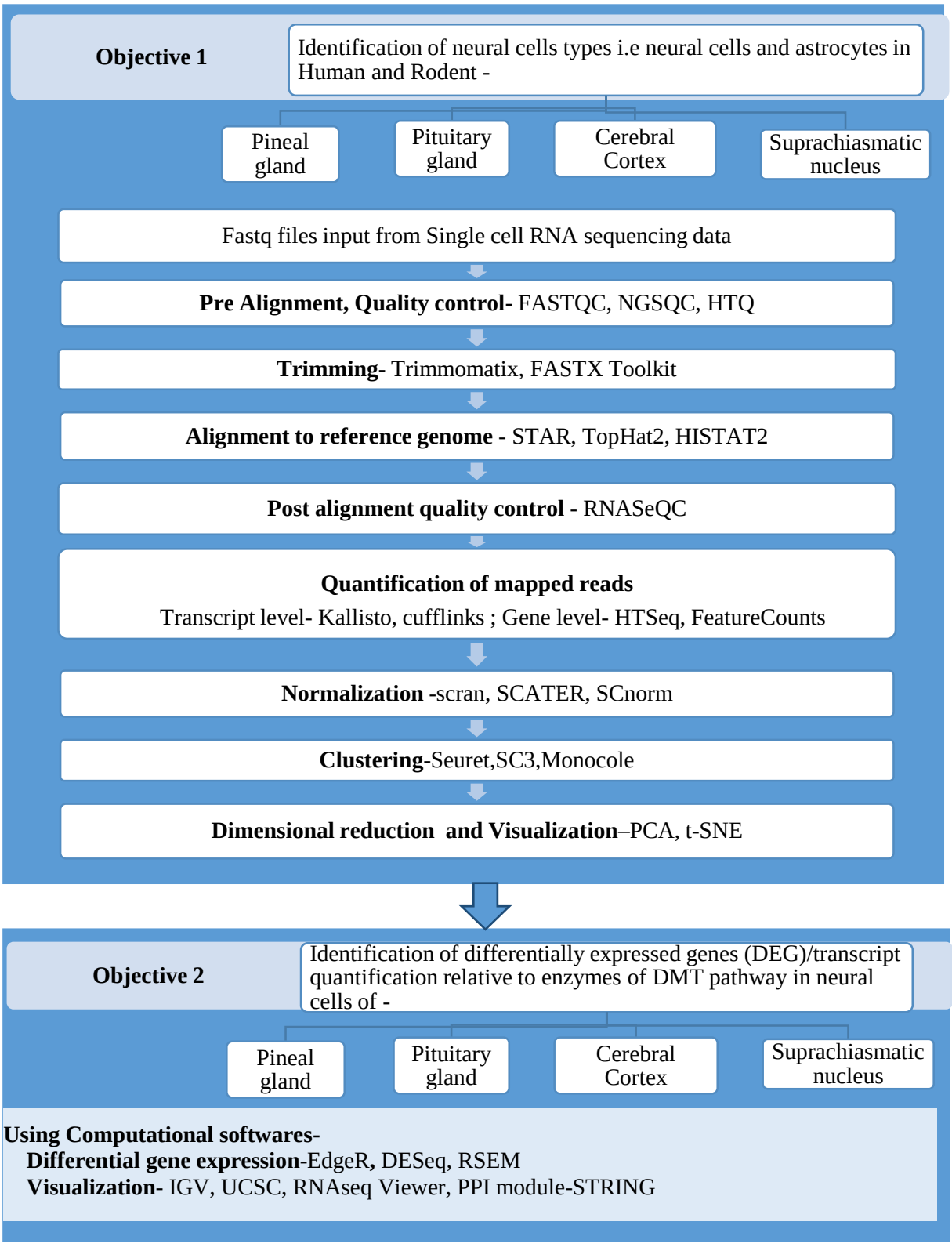
Regulatory gene network modelling and pathway construction can be done for establishing a connectome. The KEGG pathway database is a collection of pathway maps representing the current knowledge on the molecular interaction, reaction and relation networks (Kanehisa et al., 2017). Pathview, KEGG and DAVID and similar bioinformatic softwares will be used for pathway analysis softwares like jActiveModule and Cytoscape will be used for network analysis (Rahman et al., 2020).

5. Objective - Correlation with neurocognitive behaviour/states and consciousness processing

With relation to cognition, co-expression with genes expressed highly in cognitive activities or

knowledge from databases like BrainEXP (Jiao et al., 2019) and BrainMap for brain functional connectivity or fMRI or EEG scans will be integrated with the results obtained from this insilico study.

PLAN OF WORK


Objective 1
Identification of neural cells types i.e neural cells and astrocytes in Human and Rodent -
Pineal gland
Pituitary gland
Cerebral Cortex
Suprachiasmatic nucleus
Fastq files input from Single cell RNA sequencing data
Pre Alignment, Quality control- FASTQC, NGSQC, HTQ
Trimming- Trimmomatix, FASTX Toolkit
Alignment to reference genome - STAR, TopHat2, HISTAT2
Post alignment quality control - RNASeQC
Quantification of mapped reads
Transcript level- Kallisto, cufflinks ; Gene level- HTSeq, FeatureCounts
Normalization -scran, SCATER, SCnorm
Clustering-Seuret,SC3,Monocole
Dimensional reduction and Visualization–PCA, t-SNE
Objective 2
Identification of differentially expressed genes (DEG)/transcript quantification relative to enzymes of DMT pathway in neural cells of -
Pineal gland
Pituitary gland
Cerebral Cortex
Suprachiasmatic nucleus
Using Computational softwares-
Differential gene expression-EdgeR, DESeq, RSEM
Visualization- IGV, UCSC, RNAseq Viewer, PPI module-STRING

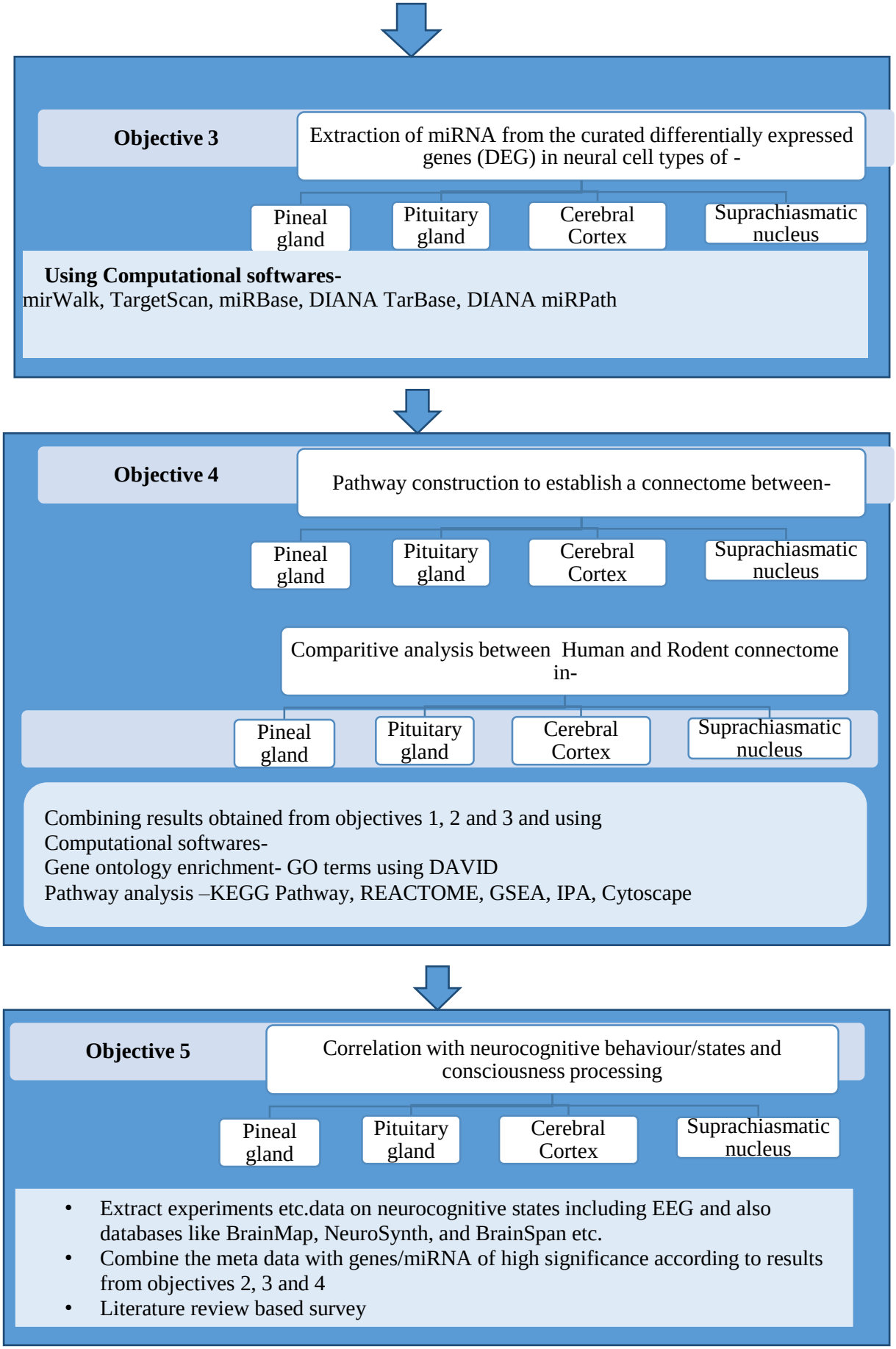

Objective 3
Extraction of miRNA from the curated differentially expressed genes (DEG) in neural cell types of -
Pineal gland
Pituitary gland
Cerebral Cortex
Suprachiasmatic nucleus
Using Computational softwares-
mirWalk, TargetScan, miRBase, DIANA TarBase, DIANA miRPath
Objective 4
Pathway construction to establish a connectome between-
Pineal gland
Pituitary gland
Cerebral Cortex
Suprachiasmatic nucleus
Comparitive analysis between Human and Rodent connectome in-
Pineal gland
Pituitary gland
Cerebral Cortex
Suprachiasmatic nucleus
Combining results obtained from objectives 1, 2 and 3 and using
Computational softwares-
Gene ontology enrichment- GO terms using DAVID
Pathway analysis –KEGG Pathway, REACTOME, GSEA, IPA, Cytoscape
Objective 5
Correlation with neurocognitive behaviour/states and consciousness processing
Pineal gland
Pituitary gland
Cerebral Cortex
Suprachiasmatic nucleus
• Extract experiments etc.data on neurocognitive states including EEG and also databases like BrainMap, NeuroSynth, and BrainSpan etc.
• Combine the meta data with genes/miRNA of high significance according to results from objectives 2, 3 and 4
• Literature review based survey

Fig 1 : The proposed plan of work.

PICTORIAL REPRESENTATION

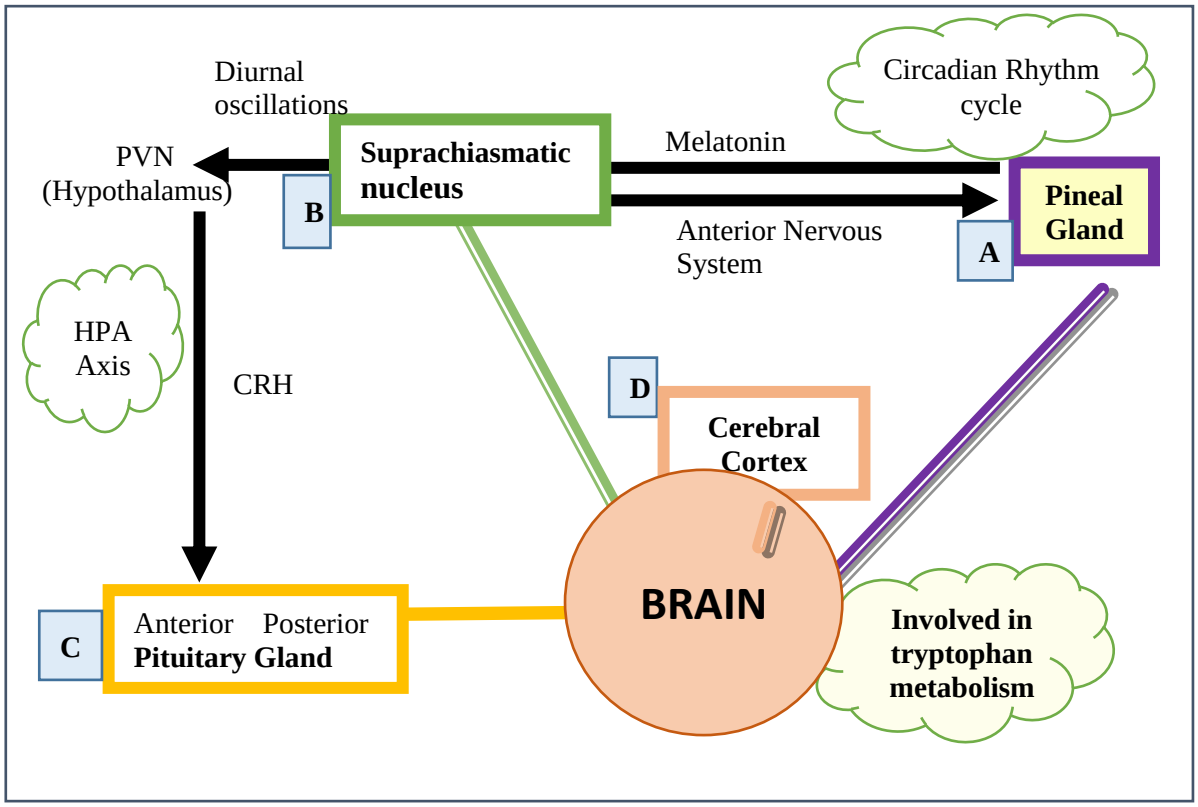


Fig 2 : Brain Quartet and Interconnectivity showing interconnections betweeen Pineal gland, Pituitary gland, Suprachiasmatic nucleus and Cerebral cortex

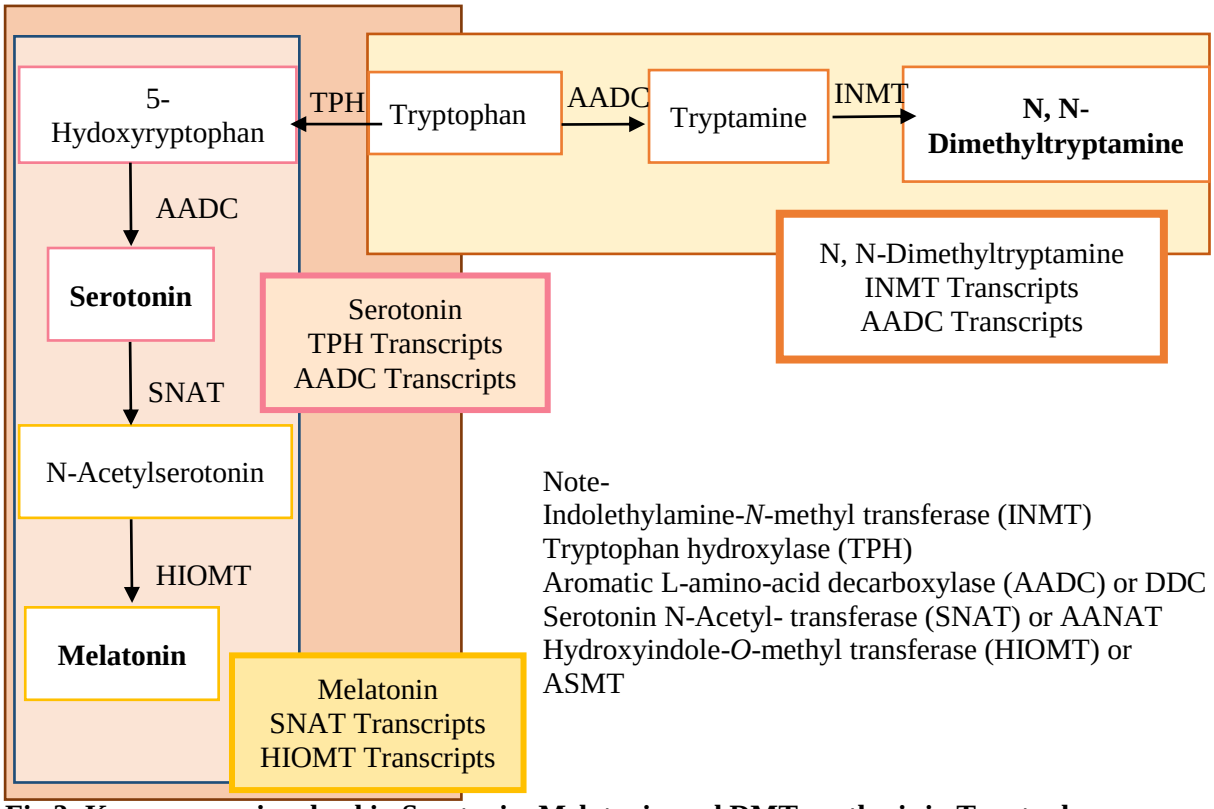


Fig 3: Key enzymes involved in Serotonin, Melatonin and DMT synthesis in Tryptophan metabolism

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